

04/29684

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 5/10, A61K 39/39, 48/00		A1	(11) International Publication Number: WO 99/38954
			(43) International Publication Date: 5 August 1999 (05.08.99)
(21) International Application Number: PCT/US99/02253			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
(22) International Filing Date: 2 February 1999 (02.02.99)			
(30) Priority Data: 60/073,405 2 February 1998 (02.02.98) US			
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(54) Title: A UNIVERSAL IMMUNOMODULATORY CYTOKINE-EXPRESSING BYSTANDER CELL LINE AND RELATED COMPOSITIONS AND METHODS OF MANUFACTURE AND USE			
(57) Abstract <p>The present invention provides a universal immunomodulatory cytokine-expressing bystander cell line, a composition comprising such a cell line and a cancer antigen, a method of making such a cell line, and a method of using such a composition.</p>			

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**A UNIVERSAL IMMUNOMODULATORY CYTOKINE-EXPRESSING
BYSTANDER CELL LINE AND RELATED COMPOSITIONS AND
METHODS OF MANUFACTURE AND USE**

5 This application claims priority to copending U.S. provisional patent application serial no. 60/073,405, which was filed February 2, 1998.

TECHNICAL FIELD OF THE INVENTION

10 The present invention relates to a universal immunomodulatory cytokine-expressing bystander cell line, a composition comprising such a cell line and a cancer antigen, a method of making such a cell line, and a method of using such a composition.

BACKGROUND OF THE INVENTION

15 Cancer immunotherapy is a therapeutic treatment of cancer. It is based on the premise that the failure of the immune system to reject spontaneously arising tumors is related to the failure of the immune system to respond appropriately to tumor antigens. In a functioning immune system, tumor antigens are processed and expressed on the cell surface in the context of major histocompatibility
20 complex (MHC) class I and II molecules, which, in humans, are also referred to as "human leukocyte associated" (HLA) molecules. When complexed to antigens, the MHC class I and II molecules are recognized by CD8⁺ and CD4⁺ T-cells, respectively. This recognition generates a set of secondary cellular signals and the paracrine release of specific cytokines or soluble so-called "biological response
25 modifiers" that mediate interactions between cells and stimulate host defenses to fight off disease. The release of cytokines then results in the proliferation of antigen-specific T-cells.

30 Active immunotherapy involves the injection of cancer or tumor cells to generate either a novel or an enhanced systemic immune response. The tumor cells employed can be autologous, i.e., derived from the host to be treated, or allogeneic, i.e., derived from a host other than the one to be treated. Such a strategy is referred to as a "vaccine," meaning use of an antigen source, such as an

intact cancer or tumor cell, to stimulate an immune response against established metastatic cancer -- not prophylactic immunization.

The use of autologous tumor cells as "vaccines" to augment anti-tumor immunity has been extensively investigated (Oettgen et al., in *Biologic Therapy of Cancer*, DeVita et al., eds. (Lippincott, Philadelphia, PA), pp. 87-119 (1991)).

Although a few patients appear to have benefited from autologous cancer vaccines, their use has only realized partial and short-lived results. Thus, numerous attempts have been made to improve the efficacy of cancer vaccines. Such attempts include radiation and/or chemical modification, infection of autologous tumor cells with virus prior to reinjection into a patient, and transfection/transduction of the tumor cells with genes encoding immunologically relevant molecules, such as cytokines or T-cell co-stimulatory molecules. These attempts, which have been initially explored in murine tumor models, have demonstrated the ability to prime systemic immune responses capable of mediating the rejection of micrometastatic tumors at distant sites. Analysis of the mechanisms of the anti-tumor immune responses generated through such vaccination has underscored the importance of the T-cell arm of the immune system in tumor rejection. Nonspecific immunostimulants also have been used, although little improvement has been realized.

At the clinical level, transfection/transduction of tumor cells with genes encoding immunologically relevant molecules involves tumor resection, culture of cells isolated from the tumor, transfection/transduction of the cultured tumor cells with a gene encoding an immunologically relevant molecule, such as a cytokine, e.g., GM-CSF, irradiation of the transfected/transduced tumor cells, and administration of the irradiated tumor cells to the patient. Tumor cells that have been genetically modified to express various factors, such as IL-4, IL-2, IFN- γ , TNF- α , G-CSF, JE, IL-7 and IL-6, have been shown to lead to rejection of the genetically modified cells in syngeneic hosts (Tepper et al., *Cell* 57: 503-512 (1989); Li et al., *Mol. Immunol.* 27: 1331-1337 (1990); Golumbek et al., *Science* 254: 713-176 (1991); Fearon et al., *Cell* 60: 397-403 (1990); Gansbacher et al., *J. Exp. Med.* 172: 1217-1224 (1990); Gansbacher et al., *Cancer Res.* 50: 7820-7825 (1990); Watanabe et al., *PNAS USA* 86: 9456-9460 (1989); Asher et al., *J.*

Immunol. 146: 3227-3234 (1991); Blankenstein et al., J. Exp. Med. 173: 1047-1052 (1991); Teng et al., PNAS USA 88: 3535-3539 (1991); Colombo et al., J. Exp. Med. 173: 889-897 (1991); Rollins et al., Mol. Cell. Biol. 11: 3125-3131 (1991); Hock et al., J. Exp. Med. 174: 1291-1298 (1991); Aoki et al., PNAS USA
5 89: 3850-3854 (1992); Porgador et al., Cancer Res. 52: 3679-3686 (1992)).

Systemic immunity has been demonstrated to increase with cells that express IL-4, IL-2, IFN- γ , TNF- α , IL-7 or IL-6 (Golumbek et al. (1991), *supra*; Porgador et al. (1992), *supra*).

Various studies comparing irradiated, cytokine-transduced autologous
10 tumor cells have demonstrated that GM-CSF-transduced autologous tumor cells are the most potent inducers of long-lasting, specific tumor immunity (Dranoff et al., PNAS USA 90: 3539-3543 (1993); see, also, Asher et al., J. Immunol. 146: 3327-3334 (1990); Sanda et al., J. of Urology 151: 622-628 (1994); Simons et al., Cancer Research 57: 1537-1546 (1997)). The efficacy of GM-CSF-transduced
15 vaccines has been demonstrated in preclinical models of melanoma, lymphoma, and cancers of the lung, colon, kidney and prostate (Dranoff et al. (1990), *supra*; Golumbek et al. (1991), *supra*; Sanda et al. (1994), *supra*; Jaffee et al., J. Immunother. 18: 1-9 (1995); Caducci et al., Cancer (Phila.) 75: 2013-2020 (1995); Vieweg et al., Cancer Res. 54: 1760-1765 (1994); Jaffee et al., J. Immunother. 19:
20 1-8 (1996); Levitsky et al., J. Immunol. 156: 3858-3865 (1996)). At the site of vaccination, GM-CSF locally activates (paracrine) antigen-presenting cells (APCs), including dendritic cells and macrophages. APCs subsequently prime CD4⁺ and CD8⁺ T-cells, which recognize tumor-associated antigens at metastatic sites, thereby mediating systemic antitumor immunity.

25 A number of phase-I clinical trials in patients with metastatic cancer have taken place. At Johns Hopkins University, patients with metastatic renal cell carcinoma were treated either with unmodified irradiated autologous tumor cells or irradiated autologous tumor cells transduced to secrete GM-CSF. Measured parameters of immunity paralleled what had been seen in the mouse models and
30 the randomization enabled a clear demonstration of the role of GM-CSF as a molecular adjuvant. A subsequent trial in the treatment of patients with metastatic

prostate cancer with autologous GM-CSF-transduced tumor cells extended these observations. Ongoing is a trial at the Dana Farber Cancer Institute in which patients with metastatic melanoma are being treated with autologous GM-CSF-transduced tumor cells.

5 The pilot studies at Johns Hopkins University and the Dana Farber Cancer Institute and elsewhere have lent support to the use of irradiated, cytokine-transduced autologous tumor vaccines as a therapeutic method of treatment. For many malignancies, large numbers of autologous tumor cells are easily obtained at presentation prior to surgery or chemotherapy-induced remission. For diseases
10 such as acute or chronic leukemias, lymphoma, and colonic carcinoma, well over 5×10^9 tumor cells can be obtained and stored with methodologies currently in use at most cancer treatment centers. However, the need for *in vitro* culture to enable gene transfer and the inability to obtain reproducibly and uniformly high levels of GM-CSF production through such procedures limits this therapeutic approach.

15 In order to circumvent this problem, a number of investigators are conducting studies of immunization with irradiated, GM-CSF-transfected allogeneic tumor cell lines, such as in the treatment of prostate and pancreatic cancer. The rationale for this approach is that the relevant tumor antigen(s) may be shared between the immunizing allogeneic tumor cell line and the tumor of the
20 patient who is being immunized. Given that the relevant tumor antigens have not been defined in most of these systems, this assumption remains as yet unproven.

 In view of the above, materials and methods that would obviate the need for *in vitro* culture for purposes of gene transfer to autologous tumor cells and that would enable reproducible and uniform immunomodulatory cytokine, e.g., GM-
25 CSF, production would be highly desirable. Therefore, it is an object of the present invention to provide such materials and methods. This and other objects and advantages will become apparent from the detailed description provided herein.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a universal immunomodulatory cytokine-expressing bystander cell line. The universal bystander cell line is a human cell line, which either naturally lacks major histocompatibility class I (MHC-I) antigens and major histocompatibility class II (MHC-II) antigens or is modified so that it lacks MHC-I antigens and MHC-II antigens. In addition, the universal bystander cell line is modified by introduction of a nucleic acid molecule comprising a nucleic acid sequence encoding an immunomodulatory cytokine operably linked to a promoter. Preferably, the immunomodulatory cytokine is granulocyte macrophage-colony stimulating factor (GM-CSF). The universal bystander cell line expresses preferably at least about 500 ng, more preferably at least about 1,000 ng, GM-CSF /10⁶ cells/24 hours. Alternatively and also preferably, the immunomodulatory cytokine is interleukin 2 (IL-2). Preferably, the human cell line is characterized by the absence of B-lymphocyte markers of immunoglobulin, an Epstein-Barr virus (EBV) genome and an associated nuclear antigen, and receptors for EBV. A preferred human cell line is one that is derived from a blast crisis of chronic myeloid leukemia. An example of a preferred cell line is K562. Preferably, the universal bystander cell line grows in defined, i.e., serum-free, medium. The promoter to which the nucleic acid sequence encoding an immunomodulatory cytokine is operably linked is preferably a cytomegalovirus promoter. Preferably, the universal bystander cell line further comprises a nucleic acid sequence encoding hygromycin resistance operably linked to a promoter and is selected by growth in culture medium comprising at least about 400 µg/ml hygromycin, preferably followed by growth in culture medium comprising at least about 1,000 µg/ml hygromycin.

Also provided by the present invention is a composition comprising the universal bystander cell line and a cancer antigen. A method of making a universal immunomodulatory cytokine-expressing bystander cell line is also provided, as well as a method of stimulating an immune response to a cancer in a human patient.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph of ng GM-CSF/ 10^6 cells/24 hrs versus cell line.

Figure 2A is a graph of counts versus relative fluorescence for MHC-I
5 antigen expression.

Figure 2B is a graph of counts versus relative fluorescence for MHC-II
antigen expression.

Figure 3 is a graph of percent (%) viable cells (trypan blue negative) versus
days post-irradiation.

10 Figure 4 is a graph of % tumor-free survival versus days post-tumor
challenge.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated on the observation that, in the context
15 of a cancer vaccine, the cancer cell, itself, need not directly produce an
immunomodulatory cytokine, such as GM-CSF, in order to stimulate an immune
response against the cancer cell. The present invention is further predicated on the
surprising and unexpected discovery that a universal bystander cell line, which can
locally produce an immunomodulatory cytokine, such as GM-CSF, at
20 unprecedented high levels, can be made. The present invention is advantageous in
that, through the administration to a patient of a composition comprising the
universal bystander cell and an autologous cancer antigen, e.g., an autologous
tumor cell, paracrine production of an immunomodulatory cytokine, such as GM-
CSF, adequate recruitment of APCs, and successful priming against the cancer
25 antigens are achieved, thereby obviating the need to culture and transduce
autologous tumor cells for each and every patient and to contend with variable
and, oftentimes, inefficient transduction efficiencies.

In view of the above, the present invention provides a universal,
immunomodulatory cytokine-producing bystander cell line. The cell line is a
30 mammalian, preferably a human, cell line, which naturally lacks major
histocompatibility class I (MHC-I) antigens and major histocompatibility class II

(MHC-II) antigens or is modified so that it lacks MHC-I antigens and MHC-II antigens. Theoretically, any mammalian, preferably human, cell line that is capable of paracrine production of an immunomodulatory cytokine can be used. The human cell line preferably is characterized by the absence of B-lymphocyte markers of immunoglobulin, an Epstein-Barr virus (EBV) genome and an associated nuclear antigen, and receptors for EBV. A preferred human cell line is one that is derived from a blast crisis of chronic myeloid leukemia. An example of a preferred human cell line is K562 (ATCC CCL- 243; Lozzio et al., Blood 45(3): 321-334 (1975); Klein et al., Int. J. Cancer 18: 421-431 (1976)). The universal bystander cell line preferably grows in defined, i.e., serum-free, medium. In addition, the universal bystander cell line preferably grows as a suspension.

Cells that lack MHC-I antigens can be achieved by interfering with the expression and/or transport of the α chain. Cells that lack MHC-II antigens can be achieved by interfering with expression and/or transport of the α and β chains. Inactivation of MHC-I and -II antigens can be achieved in a variety of ways (see, for example, U.S. Patent No. 5,574,205). For example, a "dominant negative" can be created. A single modified β_2 microglobulin gene, whose protein product effectively complexes with MHC-I molecules and acts as a decoy, thereby preventing the insertion of MHC-I antigens into the membrane, can be overexpressed. A similar approach can be used with respect to MHC-II antigens by overexpressing modified genes encoding defective α or β subunits that complex with the host cells' subunits rendering them nonfunctional. Transfection, retroviral infection or homologous recombination can be used to achieve expression of modified MHC or β_2 microglobulin genes or inactivation of genes.

Levels of MHC-I antigen on the cell surface can be reduced by introducing into cells a sequence encoding adenoviral E19 protein by transfection or retroviral infection. The protein forms complexes specifically with MHC-I antigens in the rough endoplasmic reticulum preventing normal transport of MHC-I molecules to the plasma membrane (Andersson et al., Cell 43: 215-222 (1985); Pabo et al., Advances in Cancer Research 42: 151-163 (1989)).

In addition to lacking MHC-I and MHC-II antigens or being modified to lack MHC-I and MHC-II antigens, the mammalian, preferably human, cell line is modified by introduction of a nucleic acid molecule comprising a nucleic acid sequence encoding an immunomodulatory cytokine operably linked to a promoter.

5 By "modified" is meant the provision to the universal bystander cell line of a nucleic acid molecule, e.g., vector, that comprises a nucleic acid sequence encoding a cytokine that either is not expressed in the cell line or, as a result of the provision of the nucleic acid molecule, is now expressed at a greater level. A "vector" encompasses a DNA molecule, such as a plasmid, virus or other vehicle,
10 which contains one or more heterologous or recombinant DNA sequences, e.g., a cytokine gene or cytokine coding sequence of interest under the control of a functional promoter and possibly also an enhancer, and that is capable of functioning as a vector as that term is understood by those of ordinary skill in the art.

Any suitable vector can be employed that is appropriate for introduction of
15 nucleic acids into eukaryotic cells, or more particularly animal cells, such as mammalian, e.g., human, cells. Preferably, the vector is compatible with the cell, e.g., is capable of imparting expression of the cytokine gene or coding sequence, and is stably maintained or relatively stably maintained in the cell. Desirably, the vector comprises an origin of replication. When a cytokine coding sequence is transferred
20 (i.e., as opposed to a cytokine gene having its own promoter), optimally the vector also contains a promoter that is capable of driving expression of the coding sequence and that is operably linked to the coding sequence. A coding sequence is "operably linked" to a promoter (e.g., when both the coding sequence and the promoter together constitute a native or recombinant cytokine gene) when the promoter is
25 capable of directing transcription of the coding sequence.

Appropriate viral vectors include, but are not limited to simian virus 40, bovine papilloma virus, Epstein-Barr virus, adenovirus, herpes virus, vaccinia virus, Moloney murine leukemia virus, Harvey murine sarcoma virus, murine mammary tumor virus, and Rous sarcoma virus. Any plasmid suitable for use in a eukaryote,
30 in particular a mammal, e.g., a human, can be used in the context of the present invention. Desirably, the plasmid comprises a promoter, such as the

cytomegalovirus promoter, an origin of replication, such as the SV40 origin of replication, a selectable marker, such as antibiotic resistance, and provides for mRNA with poly A tails. A preferred example of a plasmid is pCEP4 (See Example 1).

5 Reference to a vector or other DNA sequences as "recombinant" merely acknowledges the linkage of DNA sequences which are not typically conjoined as isolated from nature. A "gene" is any nucleic acid sequence coding for a protein or a nascent mRNA molecule. Whereas a gene comprises coding sequences and non-coding (e.g., regulatory) sequences, a "coding sequence" does not include any non-coding DNA. A "promoter" is a DNA sequence that directs the binding of RNA
10 polymerase and thereby promotes RNA synthesis. "Enhancers" are *cis*-acting elements of DNA that stimulate or inhibit transcription of adjacent genes. An enhancer that inhibits transcription also is termed a "silencer." Enhancers differ from DNA-binding sites for sequence-specific DNA binding proteins found only in
15 the promoter (which also are termed "promoter elements") in that enhancers can function in either orientation, and over distances of up to several kilobase pairs (kb), even from a position downstream of a transcribed region.

As used herein, cytokine "gene" or "coding sequence" includes cytokine genomic or cDNA sequences, greater and lesser sequences and mutations thereof,
20 whether isolated from nature or synthesized in whole or in part, as long as the gene or coding sequence can express a protein having the characteristic function of the cytokine, i.e., the ability to stimulate the host immune response. The means of modifying genes or coding sequences are well-known in the art, and also can be accomplished by means of commercially available kits (e.g., New England Biolabs,
25 Inc., Beverly, MA; Clontech, Palo Alto, CA). The cytokine gene or coding sequence can be of any suitable source, e.g., a mammalian species such as a human. Preferably, however, the cytokine gene or coding sequence comprises a GM-CSF sequence, particularly a human GM-CSF gene or coding sequence, including a human GM-CSF cDNA sequence (e.g., as described by Cantrell et al., PNAS USA
30 82: 6250-6254 (1985)).

Preferably, all proper transcription, translation and processing signals (e.g., splicing and polyadenylation signals) are correctly arranged on the vector such that the cytokine gene or coding sequence will be appropriately transcribed and translated in the cell into which it is introduced. The manipulation of such signals to ensure appropriate expression in host cells is well within the knowledge and expertise of the ordinary skilled artisan. Whereas a cytokine gene is controlled by (i.e., operably linked to) its own promoter, another promoter, including a constitutive promoter, such as, for instance the adenoviral type 2 (Ad2) or type 5 (Ad5) major late promoter (MLP) and tripartite leader, the cytomegalovirus (CMV) immediate early promoter/enhancer, the Rous sarcoma virus long terminal repeat (RSV-LTR), and others, can be employed to command expression of the cytokine coding sequence. The CMV promoter is a preferred promoter.

Alternately, a tissue-specific promoter (i.e., a promoter that is preferentially activated in a given tissue and results in expression of a gene product in the tissue where activated) can be used in the vector. Such promoters include, but are not limited to, the elastase I gene control region, which is active in pancreatic acinar cells as described by Swift et al., *Cell* 38: 639-646 (1984) and MacDonald, *Hepatology* 7: 425-515 (1987); the insulin gene control region, which is active in pancreatic beta cells as described by Hanahan, *Nature* 315: 115-122 (1985); the hepatocyte-specific promoter for albumin or α_1 -antitrypsin described by Frain et al., *Mol. Cell. Biol.* 10: 991-999 (1990), and Ciliberto et al., *Cell* 41: 531-540 (1985); and the albumin and α_1 -antitrypsin gene control regions, which are both active in liver as described by Pinkert et al., *Genes and Devel.* 1: 268-276 (1987), and Kelsey et al., *Genes and Devel.* 1: 161-171 (1987).

Similarly, a tumor-specific promoter, such as the carcinoembryonic antigen for colon carcinoma described by Schrewe et al., *Mol. Cell Biol.* 10: 2738-2748 (1990), can be used in the vector. Along the same lines, promoters that are selectively activated at different developmental stages (e.g., globin genes are differentially transcribed in embryos and adults) can be employed for gene therapy of certain types of cancer.

Another option is to use an inducible promoter, such as the IL-8 promoter, which is responsive to TNF, or the 6-16 promoter, which is responsive to interferons, or to use other similar promoters responsive to other cytokines or other factors present in a host or that can be administered exogenously. Use of a cytokine-inducible promoter has the added advantage of allowing for auto-inducible expression of a cytokine gene. According to the invention, any promoter can be altered by mutagenesis, so long as it has the desired binding capability and promoter strength.

Various methods can be employed for delivering a nucleic acid molecule, e.g., a vector, to a cell *in vitro*. For instance, such methods include electroporation, membrane fusion with liposomes, high velocity bombardment with DNA-coated microprojectiles, incubation with calcium phosphate-DNA precipitate, DEAE-dextran mediated transfection, infection with modified viral nucleic acids, direct microinjection into single cells, and the like. Other methods are available and are known to those skilled in the art.

If the universal bystander cell line is to be used in the context of cancer immunotherapy, the immunomodulatory cytokine is one that stimulates an immune response against a cancer cell or a cancer antigen, i.e., any protein, carbohydrate or other component capable of eliciting an immune response. An inhibitory cytokine or a cytokine that prevents priming cannot be used in the context of cancer immunotherapy. While the nucleic acid molecule preferably encodes a single immunomodulatory cytokine, the nucleic acid molecule can encode two or more immunomodulatory cytokines, such as cytokines that act synergistically.

Examples of suitable immunomodulatory cytokines include interferons (e.g., IFN α , IFN β and IFN γ), interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10 and IL-12), tumor necrosis factors (e.g., TNF α and TNF β), erythropoietin (EPO), FLT-3 ligand, macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF). The most preferred

immunomodulatory cytokine is GM-CSF, such as human GM-CSF. An alternatively preferred immunomodulatory cytokine is IL-2.

Desirably, the universal bystander cell line expresses unprecedented high levels of an immunomodulatory cytokine, which preferably is GM-CSF.

- 5 Preferably, the universal bystander cell line expresses at least about 500 ng GM-CSF/ 10^6 cells /24 hours. More preferably, the universal bystander cell line expresses at least about 1,000 ng GM-CSF/ 10^6 cells/24 hours.

- For purposes of identification and selection, preferably the nucleic acid molecule comprising a nucleic acid sequence encoding an immunomodulatory cytokine operably linked to a promoter further comprises a nucleic acid sequence
10 encoding a selectable marker operably linked to a promoter. Preferably, the selectable marker is an antibiotic resistance gene, such as hygromycin resistance. When the selectable marker is hygromycin resistance, preferably the universal bystander cell line is selected by growth in a culture medium comprising at least
15 about 400 μ g/ml hygromycin, more preferably at least about 1,000 μ g/ml hygromycin.

- In addition to the above, the present invention provides a composition comprising the above-described universal bystander cell line and a cancer antigen. The cancer antigen can be a cancer cell or a cancer cell-surface antigen, such as
20 one that has been recombinantly produced or immunoprecipitated. Preferably, the cancer antigen is a cancer cell, the isolation and culture of which is within the skill in the art (see, e.g., WO 97/24132, in particular Example 4). A cell-surface antigen can be used instead of a cell when the cell-surface antigen has been identified and characterized and has been determined to induce an anti-cancer
25 immune response. In this regard, the universal bystander cell line can be genetically modified to express a cancer antigen. For example, the bystander cell can be genetically modified to express MAGE for the treatment of melanoma, *ras* for the treatment of pancreatic cancer, and BCR-ABL for the treatment of chronic myelogenous leukemia.

- 30 A composition or implant appropriate for administration *in vivo* can comprise appropriate carriers or diluents, which further can be pharmaceutically acceptable.

The means of making such a composition or an implant have been described in the art, see, for instance, *Remington's Pharmaceutical Sciences*, 16th Ed., Mack, ed. (1980). Use of a balanced salt solution, such as Hanks' balanced salt solution, is preferred in the composition.

- 5 In pharmaceutical dosage form, a composition can be used alone or in appropriate association, as well as in combination, with other pharmaceutically active compounds as are known in the art.

A composition of the present invention can be provided in unit dosage form, wherein each dosage unit contains a predetermined amount of the composition,
10 alone or in appropriate combination with other active agents. The term "unit dosage form" as used herein refers to physically discrete units suitable as unitary dosages for human and other mammalian subjects, each unit containing a predetermined quantity of the composition of the present invention, alone or in combination with another active agent, calculated in an amount sufficient to produce the desired effect, in
15 association with a pharmaceutically acceptable diluent, carrier, or vehicle, where appropriate. The specifications for the novel unit dosage forms of the present invention depend on the particular pharmacodynamics associated with the pharmaceutical composition in the particular host.

The present invention also provides a method of making a universal
20 immunomodulatory cytokine-expressing bystander cell line. In one embodiment, the method comprises (i) obtaining a mammalian, preferably a human, cell line that does not express MHC-I antigens and MHC-II antigens, (ii) modifying the mammalian, preferably human, cell line by introducing into the mammalian, preferably human, cell line a nucleic acid molecule comprising a nucleic acid
25 sequence encoding an immunomodulatory cytokine operably linked to a promoter and a nucleic acid sequence encoding a selectable marker operably linked to a promoter, and (iii) using the selectable marker to isolate cells that produce at least about 500 ng of said immunomodulatory cytokine/ 10^6 cells/24 hours. In another embodiment, the method comprises (i) obtaining a mammalian, preferably human,
30 cell line, (ii) modifying the mammalian, preferably human, cell line so that it does not express MHC-I antigens and MHC-II antigens, (iii) further modifying the

mammalian, preferably human, cell line by introducing into the mammalian, preferably human, cell line a nucleic acid molecule comprising a nucleic acid sequence encoding an immunomodulatory cytokine operably linked to a promoter and a nucleic acid sequence encoding a selectable marker operably linked to a promoter; and (iv) using the selectable marker to isolate cells that produce at least about 500 ng of said immunomodulatory cytokine/ 10^6 cells/24 hours.

The nucleic acid molecule comprising a nucleic acid sequence encoding an immunomodulatory cytokine operably linked to a promoter and a nucleic acid sequence encoding a selectable marker operably linked to a promoter can be any nucleic acid molecule suitable for gene transfer as described above. The retroviral MFG vector, which is described in U.S. Patent No. 5,637,483, allows for rapid screening of a large number of potential immunomodulators for systemic immunity effects and assessment of the activity of complex combinations of molecules. It also provides high titer and high gene expression. Other retroviral vectors that can be used include pLJ, pEm and α SGC (see U.S. Patent No. 5,637,483, in particular Example 12). Any immunomodulatory cytokine that stimulates an anti-tumor immune response can be used (see U.S. Patent No. 5,637,483 for assays). The most preferred immunomodulatory cytokine is GM-CSF. An alternatively preferred immunomodulatory cytokine is IL-2. While any selectable marker can be used, preferably the selectable marker is an antibiotic resistance gene, such as hygromycin resistance, in which case the modified mammalian, preferably human, cell line is cultured in culture medium comprising at least about 400 μ g hygromycin/ml culture medium. More preferably, the modified mammalian, preferably human, cell line is subsequently cultured in culture medium comprising at least about 1,000 μ g hygromycin/ml culture medium. The culture medium preferably is defined, i.e., serum-free. A preferred promoter for expression of the immunomodulatory cytokine in the method is a cytomegalovirus promoter.

Further provided by the present invention is a method of stimulating an immune response to a cancer in a mammalian, preferably a human, patient. Desirably, the method effects a systemic immune response, i.e., a T-cell response,

to the cancer. The method comprises administering to the patient the above-described composition, wherein the universal bystander cell line is derived from a mammalian, preferably human, cell line, the cancer antigen is an antigen of the cancer in the patient, and the composition is rendered proliferation incompetent, such as by irradiation. Upon administration of the composition, an immune response to the cancer is stimulated.

"Administering" means the actual physical introduction of the composition into the host. Any and all methods of introducing the composition into the host are contemplated according to the invention; the method is not dependent on any particular means of introduction and is not to be so construed. Means of introduction are well-known to those skilled in the art, and also are exemplified herein.

Any suitable route of administration can be used. Preferably, the composition is administered subcutaneously or intratumorally. One skilled in the art will recognize that, although more than one route can be used for administration, a particular route can provide a more immediate and more effective reaction than another route. Local or systemic delivery can be accomplished by administration comprising application or instillation of the formulation into body cavities, inhalation or insufflation of an aerosol, or by parenteral introduction, comprising intramuscular, intravenous, intraportal, intrahepatic, peritoneal, subcutaneous, or intradermal administration. In the event that the tumor is in the central nervous system, the composition must be administered intratumorally because there is no priming of the immune system in the central nervous system.

Desirably, the immunomodulatory cytokine is derived from a human, although a cytokine from a nonhuman source can be used if it is substantially homologous to the human cytokine and has been demonstrated to exhibit similar activity. Preferably, the cancer antigen is a cell of the cancer to be treated, i.e., an autologous cancer cell. If the composition is rendered proliferation-incompetent by irradiation, typically, the universal bystander cells and the cancer cells are plated in a tissue culture plate and irradiated at room temperature using a ^{137}Cs source. Preferably, the cells are irradiated at a dose rate of from about 50 to about 200

5 rads/min, even more preferably, from about 120 to about 140 rads/min. Preferably, the cells are irradiated with a total dose sufficient to inhibit the majority of cells, i.e., preferably about 100% of the cells, from proliferating *in vitro*. Thus, desirably the cells are irradiated with a total dose of from about 10,000 to 20,000 rads, optimally, with about 15,000 rads.

Moreover, the cancer antigen, e.g., a cell of the cancer to be treated, i.e., an autologous cancer cell, optimally is treated prior to administration to enhance its immunogenicity. Preferably, this treatment comprises, as described herein, further genetic manipulation, such as, for instance, introduction of other cytokine or immune
10 co-stimulatory functions, or, for example, admixture with nonspecific adjuvants including but not limited to Freund's complete or incomplete adjuvant, emulsions comprised of bacterial and mycobacterial cell wall components, and the like.

In general, the concentration of autologous cancer cells should be sufficient to recruit APCs to the site and result in a greater immune response to the cancer to be
15 treated than would otherwise result in the absence of such treatment. Preferably, at least from about 1×10^6 to about 1×10^9 cancer cells, even more preferably, from about 1×10^7 to about 5×10^8 cancer cells are used. However, more or less cells can be used depending on the route of administration and the presence of other active agents; etc.

20 The ratio of bystander cells to autologous cancer cells in a given administration should be such that a benefit due to the presence of the immunomodulatory cytokine-producing bystander cell is realized. With respect to GM-CSF-producing bystander cells, the ratio of bystander cells to autologous cancer cells in a given administration should be such that at least 36 ng GM-
25 CSF/ 10^6 cells/24 hrs is produced. Anti-cancer immunity falls off if the quantity of GM-CSF is less than this. Cytokine levels above this quantity do not further enhance efficacy. In addition to the GM-CSF threshold, the ratio of bystander cells to autologous cancer cells should not be greater than 1:1; otherwise, the overall efficacy of the immune response is impaired. Appropriate ratios of
30 bystander cells to isolated cancer antigens can likewise be determined using routine methods in the art.

One skilled in the art also is aware of means to monitor a therapeutic (i.e., systemic immune) response upon administering a composition of the present invention. In particular, the therapeutic response can be assessed by monitoring attenuation of tumor growth and/or tumor regression. The attenuation of tumor growth or tumor regression in response to treatment can be monitored using several end-points known to those skilled in the art including, for instance, number of tumors, tumor mass or size, or reduction/prevention of metastasis. These described methods are by no means all-inclusive, and further methods to suit the specific application will be apparent to the ordinary skilled artisan.

Any type of cancer can be treated in accordance with the present inventive method. "Cancer" as used herein includes cancers, in particular those of epithelial origin, characterized by abnormal cellular proliferation and the absence of contact inhibition, which can be evidenced by tumor formation. The term encompasses cancer localized in tumors, as well as cancer not localized in tumors, such as, for instance, cancer cells that expand from a tumor locally by invasion. Thus, the method has applicability as a local adjuvant therapy for resected cancers as well as a local control of tumor growth, such as carcinomas of the bladder, breast, colon, kidney, liver, lung, ovary, pancreas, rectum and stomach, and as a treatment of a sarcoma, e.g., fibrosarcoma or rhabdosarcoma, a hematopoietic tumor of lymphoid or myeloid lineage, or another tumor, including, but not limited to, a melanoma, teratocarcinoma, neuroblastoma, or glioma.

The method of the present invention can be combined with other methods of cancer treatment. Examples of such methods include radiation, surgery and chemotherapy. In addition, the method of the present invention can be adapted for non-human mammals, for example, by employing a nonhuman mammalian cell line to generate the universal bystander cell line and a non-human mammalian source of an immunomodulatory cytokine.

The immunomodulatory cytokine-expressing bystander cell line of the present invention also can be used to suppress autoimmune disease, e.g., rheumatoid arthritis, multiple sclerosis; etc. In addition, the bystander cell line of the present invention can be used to enhance an immune response to an infectious

disease, such as HIV infection, AIDS and malaria; etc., graft vs. host rejection, and graft rejection.

EXAMPLES

- 5 The following examples serve to illustrate the present invention and are not intended to limit its scope.

Example 1

- 10 This example describes the manufacture of a universal immunomodulatory cytokine-expressing bystander cell line.

- The human GM-CSF gene was cloned by PCR from human peripheral blood. The PCR product was cloned into the *Hin* dIII-*Not* I sites in the vector pCEP4 (Invitrogen, Carlsbad, CA), which utilizes the human CMV promoter and also encodes hygromycin resistance as a selectable marker. The EBNA-1 portion
15 of this construct was excised by digestion with the restriction enzymes *Cla* I and *Avr* II.

- The linearized plasmid was used to electroporate the human cell line K562. Drug-resistant cells were initially selected in the presence of hygromycin at 400 μ g/ml. After stable transfectants were obtained, the bulk culture was assessed for
20 the production of human GM-CSF using an ELISA assay. The GM-CSF-producing bulk culture was then selected in increasing concentrations of hygromycin, up to a maximal dose of 1200 μ g/ml. Cells that were resistant to the high dose of hygromycin were subcloned in the presence of 1200 μ g/ml hygromycin. Individual subclones were expanded and then tested for the quantity
25 of GM-CSF produced per million cells per 24 hours by ELISA, using the R&D Quantikine Kit (R & D, Minneapolis, MN). The results are shown in Fig.1, which is a bar graph of ng GM-CSF/ 10^6 cells/24 hrs versus cell line. The subclones of K562 produced in excess of 1,000 ng/ 10^6 cells/24 hrs. Subclones that produced the highest quantities of GM-CSF on a per cell basis were subsequently adapted to
30 culture in 100% AIM-V medium (Life Technologies/GIBCO, Gaithersburg, MD)

in the absence of any fetal bovine serum. Cells continued to produce GM-CSF for at least four days after irradiation.

The resulting cell populations were characterized with regard to the expression of HLA Class I and Class II molecules. GM-CSF-expressing K562 cells and cells obtained from a human prostate carcinoma cell line (obtained from a patient and immortalized; Pro 22, Johns Hopkins University, Baltimore MD) were cultured in either medium alone or medium supplemented with human recombinant IFN γ (100 units/ml x 24 hrs). Cells were stained with the primary monoclonal antibodies W632 (anti-human class I heavy chain), L243 (anti-human class II), or mAb14.4.4 (anti-mouse I-E^d, an isotype-matched irrelevant control antibody). Cells were then stained with the secondary antibody goat anti-mouse IgG2aFITC (Caltag, Burlingame, CA). Ten thousand gated events were collected on a FACScan (Becton Dickinson, San Jose, CA) and data were analyzed using the CellQuest software package. The differences in expression in MHC-I and MHC-II antigens between the GM-CSF-expressing K562 cells and the Pro 22 cells are shown in Fig. 2A and Fig. 2B, respectively, which are graphs of counts versus relative fluorescence.

The resulting cell populations were also characterized for their sensitivity to ionizing radiation. GM-CSF-expressing K562 cells were irradiated with either 10,000 or 15,000 rads via a cesium gamma irradiator and then 2.5×10^6 cells of each were placed in culture in 15 mls of medium. Cells were counted and the percent of trypan blue negative cells was recorded. The results are shown in Fig. 3, which is a graph of % viable cells (trypan blue negative) versus days post-irradiation.

25

Example 2

This example describes the ratio of universal bystander cells to autologous tumor cells to be used in a composition in accordance with the present invention.

A composition in accordance with the present invention must contain a sufficient number of bystander cells to ensure that at least 36 ng GM-CSF/ 10^6 cells/24 hrs is produced. In addition, the ratio of bystander cells to tumor cells

30

must not be greater than 1:1; otherwise, the overall efficacy of the immune response will be impaired.

BALB/c mice were injected intravenously with 1×10^5 live A20 wild-type cells (NCI, Bethesda, MD) on day zero. Five days later, mice were immunized subcutaneously with the composition indicated in Fig. 4, which is a graph of % tumor-free survival versus days post-tumor challenge. Allogeneic bystander cells were derived from a C3H (H-2k) lymphoma transduced with a retrovirus (MFG) encoding mouse GM-CSF, which produced 100 ng/ 10^6 cells/24 hrs. A20 cells were transduced with the same construct, resulting in 130 ng GM-CSF/ 10^6 cells/24 hrs. Cells used in all compositions were irradiated with 5,000 rads prior to injection.

As shown in Fig. 4, subclones of GM-CSF-producing K562 cells produced in excess of 1,000 ng/ 10^6 cells/24 hrs. The use of such subclones enables the use of as few as one bystander cell per 10 autologous tumor cells with a clear margin of safety above the GM-CSF threshold of 36 ng GM-CSF/ 10^6 cells/24 hrs, by targeting 100 ng/ 10^6 cells/24 hrs.

All of the references cited herein, including patents, patent applications and publications, are hereby incorporated in their entireties by reference.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments can be used and that it is intended that the invention can be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. A universal bystander cell line, which:
 - (i) is a human cell line,
 - (ii) naturally lacks major histocompatibility class I (MHC-I) antigens and major histocompatibility class II (MHC-II) antigens or is modified so that it lacks MHC-I antigens and MHC-II antigens, and
 - (iii) is modified by introduction of a nucleic acid molecule comprising a nucleic acid sequence encoding granulocyte macrophage-colony stimulating factor (GM-CSF) operably linked to a promoter,wherein said universal bystander cell line expresses at least about 500 ng GM-CSF/ 10^6 cells/24 hours.
2. The universal bystander cell line of claim 1, wherein said human cell line is characterized by the absence of B-lymphocyte markers of immunoglobulin, an Epstein-Barr virus (EBV) genome and an associated nuclear antigen, and receptors for EBV.
3. The universal bystander cell line of claim 1, wherein said human cell line is derived from a blast crisis of chronic myeloid leukemia.
4. The universal bystander cell line of claim 1, wherein said human cell line is K562.
5. The universal bystander cell line of any of claims 1-4, which expresses at least about 1,000 ng GM-CSF/ 10^6 cells/24 hours.
6. The universal bystander cell line of any of claims 1-5, which grows in defined medium.

7. The universal bystander cell line of any of claims 1-6, wherein said promoter is a cytomegalovirus promoter.

5 8. The universal bystander cell line of any of claims 1-7, wherein said nucleic acid molecule further comprises a nucleic acid sequence encoding hygromycin resistance operably linked to a promoter and said
10 universal bystander cell line is selected by growth in a culture medium comprising at least about 400 µg/ml hygromycin.

9. The universal bystander cell line of claim 8, wherein said universal bystander cell line is selected by
15 growth in a culture medium comprising at least about 1,000 µg/ml hygromycin.

10. A composition comprising:
20 (a) a universal bystander cell line, which
(i) is a human cell line, (ii) naturally lacks MHC-I antigens and MHC-II antigens or is modified so that it lacks MHC-I antigens and MHC-II antigens, and (iii) is modified by introduction of a nucleic acid molecule comprising a nucleic acid sequence encoding an
25 immunomodulatory cytokine operably linked to a promoter, and
(b) a cancer antigen.

11. The composition of claim 11, wherein said
30 immunomodulatory cytokine is interleukin-2 (IL-2).

12. A composition comprising the universal bystander cell line of any of claims 1-9 and a cancer antigen.

13. A method of making a universal GM-CSF-expressing bystander cell line, which method comprises:

(i) obtaining a human cell line that lacks MHC-I antigens and MHC-II antigens;

5 (ii) modifying said human cell line by introducing into said human cell line a nucleic acid molecule comprising a nucleic acid sequence encoding GM-CSF operably linked to a promoter and a nucleic acid sequence encoding a selectable marker operably linked to a
10 promoter; and

(iii) using the selectable marker to isolate cells that produce at least about 500 ng of said GM-CSF/ 10^6 cells/24 hours.

15 14. A method of making a universal GM-CSF-expressing bystander cell line, which method comprises:

(i) obtaining a human cell line;

(ii) modifying said human cell line so that it lacks MHC-I antigens and MHC-II antigens;

20 (iii) further modifying said human cell line by introducing into said human cell line a nucleic acid molecule comprising a nucleic acid sequence encoding GM-CSF operably linked to a promoter and a nucleic acid sequence encoding a selectable marker operably linked to
25 a promoter; and

(iv) using the selectable marker to isolate cells that produce at least about 500 ng of GM-CSF/ 10^6 cells/24 hours.

30 15. The method of claim 13 or 14, wherein said selectable marker is hygromycin resistance.

16. The method of claim 15, wherein the modified human cell line is cultured in culture medium comprising
35 at least about 400 μ g hygromycin/ml culture medium.

17. The method of claim 16, wherein the modified human cell line is subsequently cultured in culture medium comprising at least about 1,000 µg hygromycin/ml culture medium.

18. The method of any of claims 13-17, wherein said culture medium is defined.

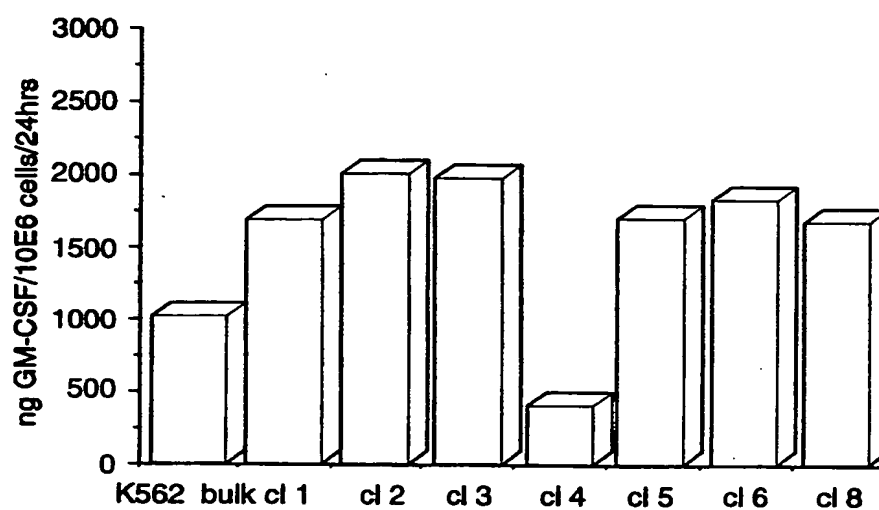
19. The method of any of claims 13-18, wherein the promoter to which the nucleic acid sequence encoding GM-CSF is operably linked is a cytomegalovirus promoter.

20. A method of stimulating an immune response to a cancer in a human patient, which method comprises administering to said patient the composition of any of claims 10-12, wherein said cancer antigen is an antigen of said cancer and wherein said composition is irradiated,

whereupon administration of said composition, an immune response to said cancer is stimulated.

21. The method of claim 20, wherein said cancer antigen is a cell of said cancer.

1/4

**FIG. 1**

2/4

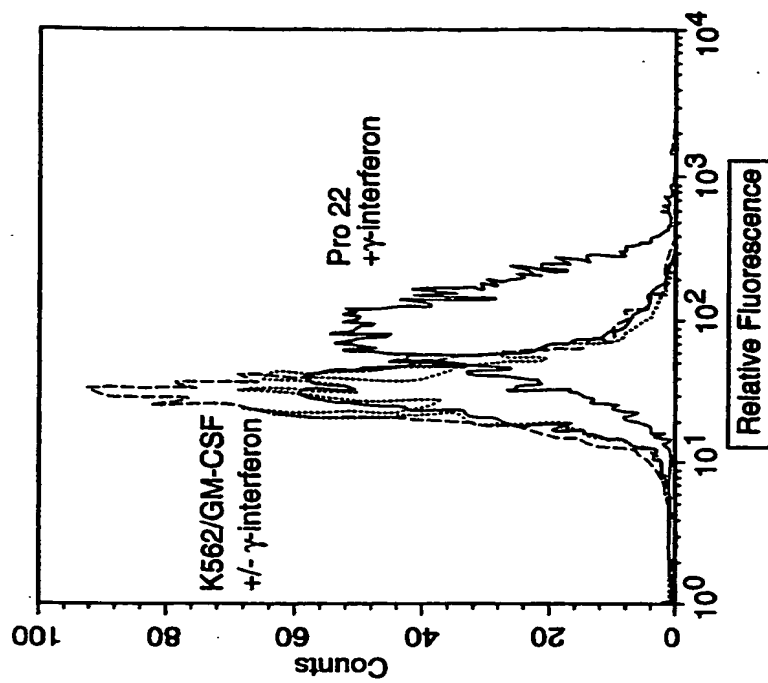


FIG. 2B

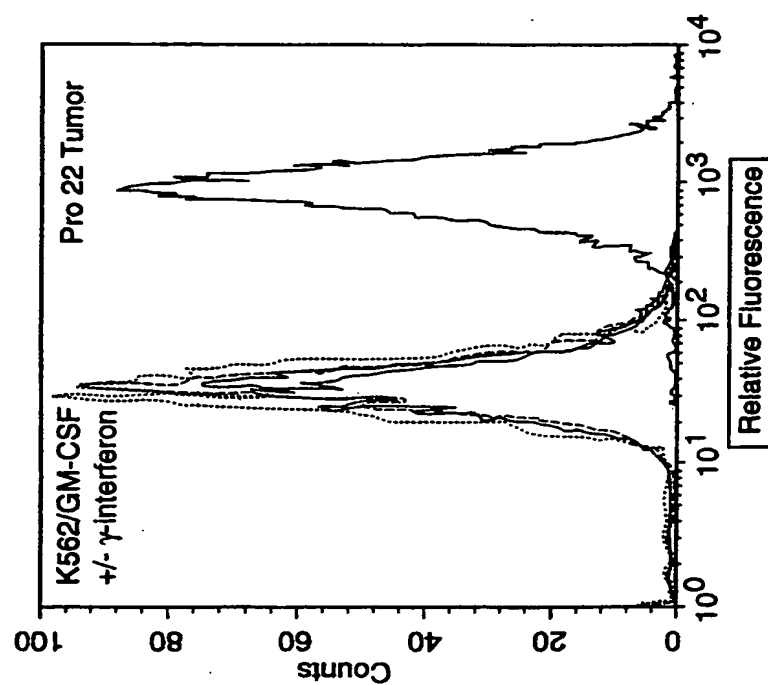
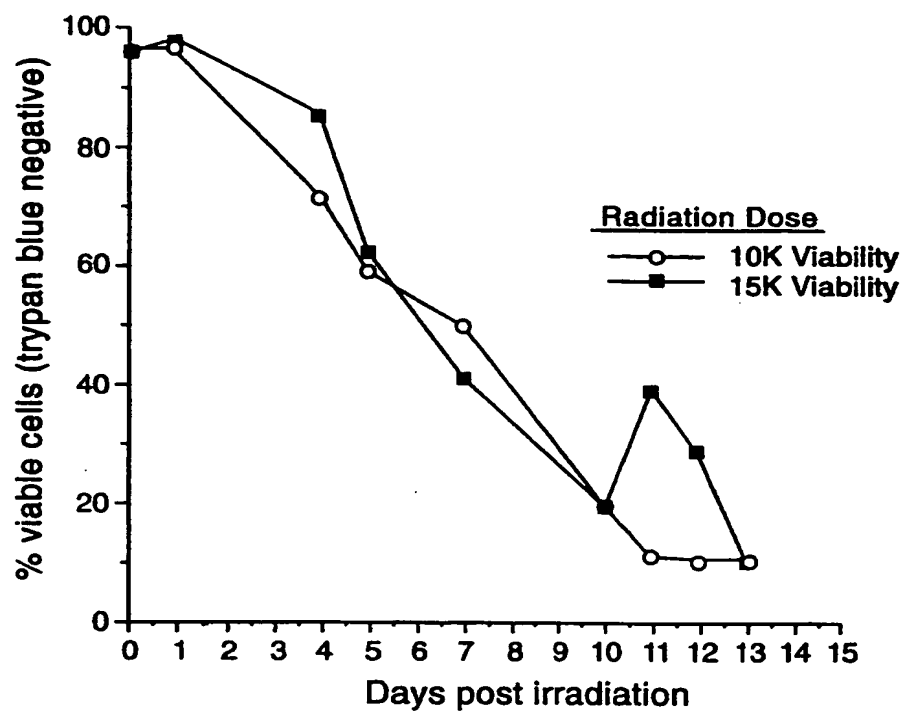


FIG. 2A

3/4

**FIG. 3**

4/4

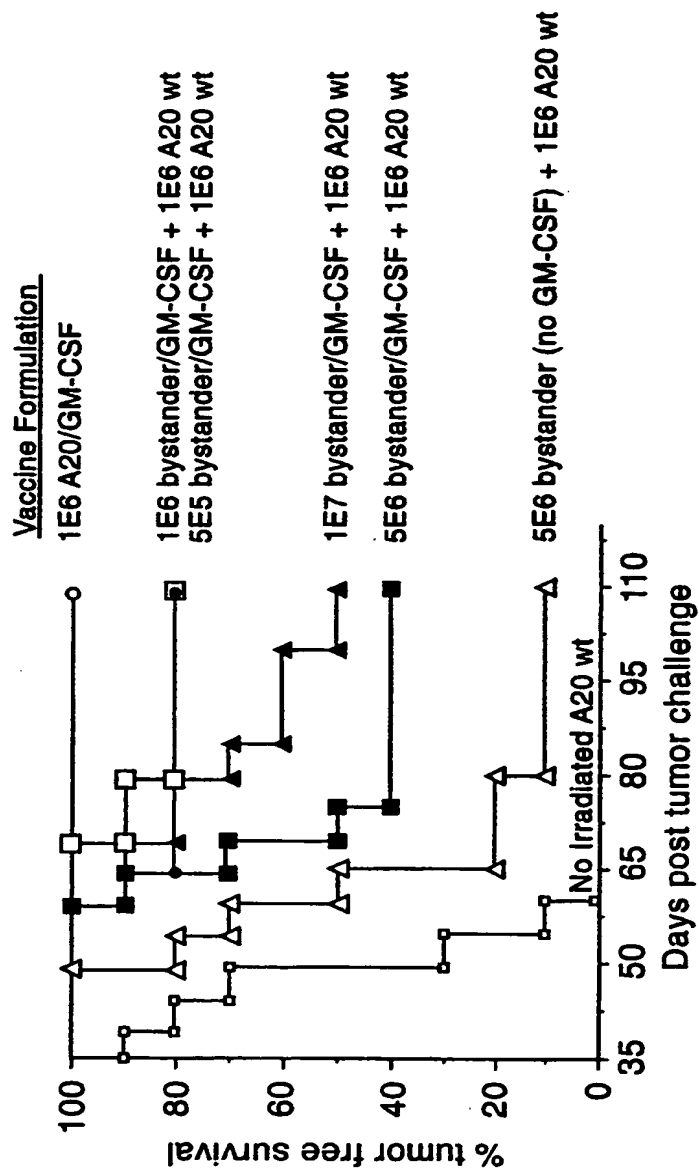


FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/02253

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N5/10 A61K39/39 A61K48/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 A61K C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DRANOFF G ET AL: "VACCINATION WITH IRRADIATED TUMOR CELLS ENGINEERED TO SECRETE MURINE GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR STIMULATE POTENT, SPECIFIC, AND LONG-LASTING ANTI-TUMOR IMMUNITY"</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, no. 8, 15 April 1993, pages 3539-3543, XP002054395 cited in the application see the whole document especially page 354 right column first paragraph</p> <p style="text-align: center;">-- --</p>	1, 10, 13, 14, 20, 21
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
<div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center;">2 July 1999</div>		Date of mailing of the international search report <div style="text-align: center;">15/07/1999</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer <div style="text-align: center;">Le Cornec, N</div>

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 99/02253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	A. MACKENSEN ET AL: "Induction of tumor-specific cytotoxic T lymphocytes by immunization with autologous tumor cells and Interleukin-2 transfected fibroblasts" JOURNAL OF MOLECULAR MEDICINE, vol. 75, no. 4, April 1997, pages 290-296, XP002108025 see the whole document	1,10-14, 20,21
A	P.T. GOLOMBEK ET AL: "Controlled release, biodegradable cytokine depots: a new approach in cancer vaccine design" CANCER RESEARCH., vol. 53, 15 December 1993, pages 5841-5844, XP002108026 MD US	
A	H.I. LEVITSKY ET AL: "Immunization with granulocyte-macrophage colony-Stimulating factor-transduced, but not B7-1-transduced, lymphoma cells primes idiotypic-specific T cells and generates potent systemic Antitumor immunity" JOURNAL OF IMMUNOLOGY., vol. 156, 1996, pages 3858-3865, XP002108027 BALTIMORE US cited in the application see the whole document	1,10,13, 14,20,21
A	A. PORGADOR ET AL: "Interleukin-6 gene transfection into lewis lung carcinoma cells suppresses the malignant phenotype and confers immunotherapeutic competence against parenteral metastatic cells" CANCER RESEARCH., vol. 52, 1 July 1992, pages 3679-3686, XP002108028 MD US cited in the application see the whole document	1,10,13, 20
A	WO 96 33746 A (GENETIC THERAPY INC) 31 October 1996 see page 15, paragraph 3 - page 16 see examples 12-14 see claims see page 5, line 9 - line 15	1,10,13, 14,20
A	WO 91 01140 A (CELL GENESYS INC) 7 February 1991 see claims & US 5 574 205 A cited in the application	14

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/02253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 07906 A (SAN DIEGO REGIONAL CANCER CENT) 29 April 1993 see claims; examples	1,10-14, 20,21
P,X	I. BORRELLO ET AL: "Description of a universal GM-CSF-producing bystander cell for use in autologous tumor cell vaccine formulations" BLOOD, vol. 92, no. 10 suppl.1 part 1-2, 15 November 1998, page 247A XP002108029 see abstract 1007	1-21

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/02253

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 20-21
are directed to a method of treatment of the human/animal
body, (rule 39.1 (IV) PCT) the search has been carried out and based
on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/02253

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9633746 A	31-10-1996	AU 5634696 A	18-11-1996
WO 9101140 A	07-02-1991	AU 633958 B	11-02-1993
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		JP 7503455 T	13-04-1995
		US 5681562 A	28-10-1997